Efficacy of fatty acids and terpenoids and weakness of electronic nose response as tracers of Asiago d'Allevo PDO cheese produced in different seasons

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EFFICACY OF FATTY ACIDS AND TERPENOIDS AND WEAKNESS OF ELECTRONIC NOSE RESPONSE AS TRACERS OF ASIAGO D'ALLEVO PDO CHEESE PRODUCED IN DIFFERENT SEASONS

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Abstract – The goal of this study was to verify the suitability of fatty acids, terpenoids and electronic nose response as potential tracers of the seasonal variations of Asiago d'Allevo PDO cheese. Cheese samples produced during the early and late summer grazing and the autumn/winter indoor seasons were compared. Based on their FA composition, the early and late summer cheeses were almost completely undistinguishable from each other. However, they both demonstrated higher quality if compared to the autumn/winter ones, showing lower levels of hypercholesterolemic saturated FAs (C12, C14, and C16) and higher levels of total mono- and polyunsaturated FAs, oleic (C18:1 *c*9), *trans*-vaccenic (C18:1 *t*11), rumenic (C18:2 *c*9*t*11), and α-linolenic (C18:3 *c*9*c*12*c*15) acids. Among terpenoids, camphene, cedrane, and total sesquiterpenoids were able to differentiate the cheeses manufactured during the three periods of production. Moreover, α-pinene, α-thujene, β-pinene, δ-3-carene, myrtenol, dihydrocarveol isomer 1, and unidentified sesquiterpene 1 distinguished the late summer cheeses from those obtained in the other two seasons. Principal component analysis of electronic nose data showed that the Asiago cheese samples were widely dispersed in the score plot and no data clusters appeared evident. Furthermore, cross-validated linear discriminant analysis of electronic nose data showed unsatisfactory classification performance (53.8%) regarding the period of production. Our results showed that coupling FAs and terpenoids information could be suitable for tracing Asiago d'Allevo PDO cheeses according to their season of production. However, no reliable information at this level seemed to be obtainable from electronic nose response.

Key words: Asiago PDO cheese, fatty acids, terpenoids, electronic nose, seasonal changes

1 Introduction

In the Italian Alpine regions, milk from ruminants is mainly used for dairy transformation into traditional cheeses. Among these is Asiago, a typical semi-hard, half-cooked cheese made with bovine milk. Since 1996 Asiago cheese has been awarded Protected Designation of Origin (PDO) status by the European Union Commission (Commission Regulation 1996). This cheese has considerable commercial relevance, with an annual production of about two million forms, corresponding to more than 24,000 tons.

Milk used to produce Asiago PDO cheese is usually obtained from cows fed pasture with limited concentrate supplementations during the grazing season, while during the indoor season the feeding is mainly based on conserved forages and notably higher amounts of concentrates. These seasonal variations in the feeding regimen applied at farm level have been widely reported to affect the chemical and nutritional characteristics of dairy products.

The possibility of using milk constituents deriving from the ruminant metabolism (fatty acids) and secondary plant metabolites (terpenoids) as specific molecular biomarkers to trace the changes in the management conditions of herds has recently gained increasing attention in the dairy industry (Engel et al. 2007).

The fatty acid composition of milk and dairy products has been reported to be particularly affected by dietary changes. Concentrations of omega 3 (n-3) fatty

acids (FAs), *trans*-vaccenic (TVA) and rumenic (RA) acids were found to increase as grass (particularly fresh grass) replaces non-grass constituents in the diet of ruminants. For this reason, these constituents of the lipid fraction have been proposed as potential tracers of grass feeding in dairy products (Monahan et al. 2010).

Since terpenoids can be transferred from plant to milk (Viallon et al. 2000), they have also been widely reported as suitable tracers of production zone (Bugaud et al. 2001; Zeppa et al. 2005) and feeding regimen (De Noni and Battelli 2008; Revello Chion et al. 2010). Moreover, as the terpenoidic content in plants is known to increase with maturity, dairy products obtained from pasture-fed ruminants could also be distinguished on the basis of the phenological phase of the grazed swards (Revello Chion et al. 2010; Tornambè et al. 2006).

The aromatic response obtainable from electronic nose (EN) technology has also been reported as being suitable for use for traceability of dairy products (Ampuero and Bosset 2003). Compared to fatty acids and terpenoids analysis, the EN technology is more rapid and cheaper. Previous studies showed successful applications of EN in discriminating cheeses according to variety (Contarini et al. 2001), geographical origin (Pillonel et al. 2003), ripening (Trihaas et al. 2005), and shelf life (Benedetti et al. 2005). However, contradictory results were obtained in detecting seasonal variations in cheese aroma (Benedetti and Mannino 2007; O'Riordan and Delahunty 2003; Schaller et al. 1999).

Previous studies conducted on the traceability of Asiago PDO cheese showed that sesquiterpenes can be successfully used as biomarkers of cheeses produced with

milk from cows grazing on different mountain pastures (Favaro et al. 2005) and that fatty acids are able to differentiate cheeses produced in alpine farms from those produced in lowland and mountain industrialized cheese factories (Schievano et al. 2008). No studies are currently available on the possibility of using the fatty acid and terpenoidic compositions of Asiago PDO cheese and the response obtainable from EN analysis to trace the seasonal variations of this cheese, associated with changes in the management system of the herds.

The aim of this study was therefore to assess the potential of fatty acids, terpenoids and electronic nose response to discriminate Asiago d'Allevo PDO cheeses produced in different seasons. This *on-farm* research reflected the real commercial production systems of Asiago PDO cheese, since it was performed under actual herd management conditions rather than under the typical tight control of experimental conditions.

2 Materials and methods

2.1 Experimental site, animals and management conditions

Ten alpine farms were chosen in the mountain territory of the 'Altopiano dei Sette Comuni' (Veneto Region, NE Italy), located within the officially recognized geographical area of Asiago PDO cheese manufacturing. The alpine farms had a herd size ranging between 30 and 65 lactating dairy cows. The majority of the farm-raised dairy cattle (64% of total cows) belonged to high-producing mono-purpose Italian Brown and Holstein Friesian breeds. The remaining cows belonged to local dual-purpose breeds (Simmental, Rendena, Alpine Grey, and

Burlina) characterized by medium milk production levels but good functional and conformation traits which make them highly adaptable to the mountain environment. All selected alpine farms regularly operate during the grazing season only, (in accordance with an extensive management regime) by making rational use of natural resources (local forages from pasture lands). In these farms, variable amounts of concentrates (never exceeding 20% of total dry matter intake) are usually provided as dietary supplementation for lactating cows at pasture.

In the autumn and winter months, the ten selected herds were housed indoors and fed common hay-and concentrate-based diets, according to the latest Asiago PDO Cheese Production Regulations (Gazzetta Ufficiale 2006). The herds were raised in farms also located within the 'Altopiano dei Sette Comuni' territory, but in lower lands. While two of these farms processed their milk at farm level (as occurring during the grazing season), the other eight farms delivered their milk to a cooperative cheese factory.

2.2 Cheese-making procedure

All Asiago d'Allevo cheese samples were produced according to the official Asiago PDO Cheese Production Regulations (Gazzetta Ufficiale 2006). Briefly, milk from one or two milkings was collected, partially skimmed after overnight creaming at about 20°C, added to termophilic starter cultures, and subsequently coagulated with bovine rennet at 35±2°C (coagulation time: 15-30 min). The curd was cut into hazelnut-size particles and half-cooked until a temperature of 47±2°C was reached. The curd was then extracted and molded in typical Asiago forms.

Asiago d'Allevo PDO cheese has a flattened cylindrical form, measuring 30-36 cm diameter, 9-12 cm height and 8-12 kg weight. The forms were turned various times while the whey was completely removed. Salting was performed either by surface dry-salting technique or by washing with brine. Maturing occurred for four months in storage bays at 10-15°C temperature and 80-85% relative humidity.

2.3 Cheese sampling

Samples collection was carried out in such a way that the different periods of production were covered. Ten Asiago cheeses produced in June and July 2007 (corresponding to the early alpine grazing season) and ten cheeses produced in August and September 2007 (corresponding to the late alpine grazing season) were collected directly *in situ* from the ten selected alpine farms. Between October 2007 and February 2008 (corresponding to the autumn/winter indoor season), a total of nine cheeses were collected from the two farms and the cooperative cheese factory that operated during the autumn and winter months. All cheeses were collected at four months of ripening. Nine representative slices (three for fatty acids, three for terpenoids, and three for electronic nose analyses) were cut from each sampled wheel. The rind was removed, as it is not generally consumed. All samples were vacuum-packed and subsequently frozen at –20°C until analyses.

2.4 Fatty acids analysis

A composite sample was obtained from the three slices previously grounded and homogenized in a blender. Cheese total lipids were extracted according to IDF (1986). Fatty acid methyl esters (FAMEs) were prepared according to Christopherson and Glass (1969) and were separated and quantified by gas chromatography (Shimadzu GC17A, Shimadzu Corporation, Kyoto, Japan) using a CP-Sil 88 capillary column (100 m × 0.25 mm ID, 0.20 μm film thickness; Varian Inc., Lake Forest, CA, USA). The column temperature was held at 45°C for 5 min and then raised 20°C·min^{-1} to a final temperature of 195°C, where it remained for 50 min. The temperatures of the injector and the flame-ionization detector were maintained at 250 and 280°C, respectively. The injection volume was 0.1 μL, with a split ratio of 21:1. The nitrogen constant linear flow rate and its average velocity were set at 1.4 mL·min^{-1} and 22 cm·s^{-1}, respectively. Peaks were identified by comparing their retention times with pure FAME standards (Matreya Inc., Pleasant Gap, PA, USA and Restek Corporation, Bellefonte, PA, USA). A butter oil reference standard (CRM 164; Commission of the European Communities, Community Bureau of Reference, Brussels, Belgium) was periodically analyzed as a control to verify column performance and was used to estimate correction factors for short chain (C4:0 to C10:0) fatty acids. Results were expressed as g·100 g^{-1} FAMEs. Fatty acid analyses were performed in duplicate.

2.5 Terpenoids analysis

A representative sample was obtained from the three slices previously ground and homogenized in a blender. Then 15 g were weighed, added to 400 μL of 10 ppm 1,3,5-triisopropylbenzene solution as internal standard, distilled under high vacuum and cooled in liquid nitrogen.

Three mL distilled aqueous samples were put into 10 mL vial and extracted with a 2-cm (50/30 μm divinylbenzene/carboxen/polydimethylsiloxane) solid-phase microextraction (SPME) fiber (Supelco Analytical, Bellefonte, PA, USA) according to Zeppa et al. (2005) with modifications. Briefly, distillates were equilibrated in a thermostatic aluminium block at 45°C for 5 min and extracted at the same temperature for 30 min under stirring. After extraction, the fiber was introduced into the injector of a gas chromatograph coupled with a mass spectrometer (GC–MS) and maintained at 270°C for 4 min for thermal desorption of terpenoids.

GC–MS analyses were carried out on a Shimadzu GC-17A gas chromatograph equipped with a Shimadzu QP-5000 quadrupole mass spectrometer detector (Shimadzu Corporation, Kyoto, Japan). Separation was achieved using a DB-WAX capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness; J&W Scientific Inc., Folsom, CA, USA). The oven temperature was programmed at 35°C and held for 5 min, increased to 173°C at 2°C·min^{-1} for 1 min, then raised to 210°C at 15°C·min^{-1} and maintained for 5 min. Injector temperature was 270°C and splitless injection mode was adopted. Helium was used as the carrier gas at 1.0 mL·min^{-1}. The detector operated in electron impact ionization mode at 70 eV

with the GC–MS interface at 230°C, and an *m/z* scan range of 33 to 300 was collected. Compound identification was achieved by comparing mass spectra and linear retention indices (LRI) with those of authentic standards and/or with those recorded in NIST12, NIST62 (National Institute of Standards and Technology, Gaithersburg, MD, USA), and other published mass spectral databases (Adams 2001). LRI were calculated by linear interpolation relative to retention times of C5–C25 n-alkanes as external references. For each terpene, the area (arbitrary unit) of the most abundant or characteristic ion *m/z* was extracted from the total ion current (TIC) and integrated with a Class-5000 Data Station ver. 2.0 software (Shimadzu Corporation, Kyoto, Japan). A semi-quantitative analysis was performed assuming that the terpenoids have the same response factor to that of the internal standard. Analyses were performed in triplicate.

2.6 Electronic nose analysis

The gas-sensor array instrument used was a portable EN PEN2 (Airsense Analytics GmbH, Schwerin, Germany) equipped with 10 metal oxide semiconductor (MOS) sensors. For the gas-sensor measurements, three samples (one from each slice) were divided into two aliquots (approximately 2 g of cheese) and transferred to 40 mL glass vials. These were sealed with preheated (105°C) Teflon/silicon septa and open screw caps and were allowed to equilibrate at room temperature for 10 min before analysis. Thereafter the samples were incubated at 40°C for 20 min before headspace gas was pumped into the sensor chamber for 20 s at a flow rate of 150 $mL \cdot min^{-1}$. Recovery time for the sensors

was 120 s (flushing with charcoal-filtered ambient air). Samples were analyzed in random order. All analytical procedures took place in an air-conditioned laboratory.

The response of the sensors during sample measurement is a curve representing the sensors' conductivity against time. The sensor signal, in fact, is expressed by the ratio G/G0 where G is the conductance of the sensor in presence of the sample, and G0 is the conductance of the sensors in reference air. The feature extracted from the sensor signal (response) was determined by identifying the curve's peak (absolute response) and then by subtracting the baseline from that value. The ten responses represent the variables in the data matrix considered for the statistical analysis.

2.7 Statistical analysis

All statistical analyses were performed using the SPSS software (version 16.0 for Windows, SPSS Inc., Chicago, Illinois, USA).

Fatty acids data were submitted to one-way analysis of variance according to the following model: $Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, where: Y_{ij} = mean of response variable; μ = overall mean; α_i = effect of sampling period (1 = early alpine grazing; 2 = late alpine grazing; 3 = autumn/winter indoor); ε_{ij} = residual error. The assumption of equal variances was assessed by Levene's homogeneity of variance test. If such assumption did not hold, the Brown-Forsythe statistic was performed to test for the equality of group means instead of the F one. Pairwise multiple comparisons were performed to test the difference between each pair of means (Tukey's HSD

test and Tamhane's T2 in the cases of equal variances assumed or not assumed, respectively). Significance was declared at $P \leq 0.05$. Data were expressed as mean ± standard error (SE).

Terpenoids data were submitted to a Kruskal-Wallis non-parametric independent group comparison. A significant Kruskal-Wallis test was followed by a Mann-Whitney U test to compare each pair of groups. The *P*-values obtained with the latter test were adjusted according to a Bonferroni correction based on the Holm-Bonferroni method. Data were expressed as mean ± SE.

Principal Component Analysis (PCA) was applied to EN data to explore and visualize hidden patterns in the data set. PCA involves a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible, and so on. All the variables were mean-centered and scaled to equal variance prior to PCA. Furthermore, a stepwise linear discriminant analysis (LDA) was also applied to EN data. This analysis is a supervised technique of classification and assignation of a sample to a previously defined group. The classification model is built by maximizing the variance between groups and minimizing the variance within groups. The model performance is then assessed on the basis of the number of samples correctly predicted as belonging to an assigned group.

3 Results

3.1 Fatty acid composition

The FA profiles of Asiago d'Allevo PDO cheese manufactured during summer months (early and late alpine grazing seasons) and autumn/winter ones (indoor season) are presented in Table 1.

The most remarkable differences in fatty acids were detected between the grazing season (both early and late summer) and the indoor season. Considering the main FA groups, total saturated FAs were significantly higher (P≤0.001) while total mono- and polyunsaturated FAs were significantly lower (P≤0.001) in the samples produced during the autumn/winter months than those produced in summer. Among saturated FAs, notable variations were detected in the majority of short- and medium-chain FAs, while no significant differences were found for margaric (C17) and stearic (C18) acids. Considering unsaturated FAs, remarkable seasonal differences were observed in the contents of *trans*-vaccenic (C18:1 *t*11, TVA) and rumenic (C18:2 *c*9*t*11, RA) acids. Cheeses produced during both the early and late summer grazing seasons showed approximately doubled levels of both TVA and RA in comparison to the cheeses produced during the indoor season (P≤0.001). Conspicuous differences were also observed between the summer and autumn/winter cheeses in the levels of oleic (C18:1 *c*9) and α-linolenic (C18:3 *c*9*c*12*c*15, ALA) acids, both significantly higher in the summer samples (P≤0.001 and P≤0.01, respectively).

The cheeses produced during the early and late summer grazing seasons were instead almost completely undistinguishable from each other. They only differed in three minor detected FAs (13-methyltetradecanoic acid -C15 *iso*-, myristoleic -C14:1 *c*9- and arachidic -C20- acids) which were significantly higher in the cheeses produced during the late alpine grazing season.

3.2 Terpenoidic composition

The terpenoidic compositions of Asiago d'Allevo PDO cheese manufactured during the early and late alpine summer grazing seasons and during the autumn/winter indoor season are presented in Table 2.

All cheese samples showed a prevalence of monoterpenoids, whose total amount was basically ($P \leq 0.10$) more abundant (on average 2.4 times) during the grazing seasons than during the indoor season. The total sesquiterpenoid content showed highly significant differences among all three periods of production in question ($P \leq 0.001$). Total sesquiterpenoids were about 3- and 17-fold higher in the early and late grazing seasons as compared to the indoor season, respectively.

Some compounds (α-thujene, δ-3-carene, dihydrocarveol isomer 1, the unidentified monoterpene 1, the unidentified sesquiterpene 1, and β-ionone) were detected only in early and/or late summer cheeses, while others, such as the two menthone isomers, were found only in cheeses produced in the autumn/winter months.

Considering the terpenoids that were detected in all three periods of production, camphene, terpinen-4-ol, β-caryophyllene, and cedrane showed significantly

higher amounts in cheeses produced during both the early and late summer than in cheeses produced in the indoor period. Moreover, other compounds such as α-pinene, β-pinene, linalool, carvone, and myrtenol, thus not being significantly different between the cheeses produced in the early grazing and indoor seasons, showed significantly higher amounts in samples produced in late summer as opposed to those produced in autumn and winter.

Many compounds showed significant differences between cheeses produced during the early and the late summer grazing seasons. In particular, some monoterpenoids (α-pinene, camphene, β-pinene, δ-3-carene, and myrtenol) and the sesquiterpenoid cedrane significantly increased with the advance of the grazing season, reaching their maximum content in cheeses produced in August and September. This trend was then followed by a general decrease in correspondence with the indoor season. The monoterpenoid γ-terpinene was the only compound that showed, on the contrary, a significantly higher content in the early rather than late summer cheese samples ($P \leq 0.05$).

Some compounds (β-myrcene, limonene, cineol, terpinolene, camphor, bornyl acetate, α-terpineol, unidentified sesquiterpene 2, methyl salicylate, and methyl dihydrojasmonate) as well as the total "miscellaneous" were detected in all the periods of production in question, without showing any significant difference among them.

3.3 Electronic nose response

Figure 1 shows the PCA score plot of gas sensors responses. This graph represents the original samples projected on the plane formed with the two principal components (PCs). The first PC described 46.12% of sample variance; the second one described 31.89%. The sum of the first three PCs accounted for more than 90% of the total explained variance.

Among the ten MOS sensors that equipped the EN used in the present study, three sensors resulted more sensitive to the headspace of the samples. They were reported to have broad-range sensitivity, especially to aromatic compounds, hydrocarbons, and nitrogen oxides. The same three sensors were the most significant in defining PCs.

Samples were thinly dispersed in the plot area defined by the first two PCs and no data clusters appeared evident. The three Asiago groups (early alpine grazing season, late alpine grazing season, and indoor season) were widely overlapping. The results of the LDA confirmed the graphical overview obtained with the PCA. Since the number of samples was limited to allow a separation into two subsets (*training* and *test* set), classification of LDA was performed using the same data that were used to derive the discriminant function. To reduce the bias deriving from this procedure, each observation was classified using a discriminant function computed from all the other observations (full cross-validation). Even with these technical adjustments, the number of misclassifications into groups was quite high. The percent of erroneously classified samples was 29.5% for “early grazing”

group, 45.9% for the “indoor” group, and as high as 65.8% in the case of “late grazing”.

4 Discussion

During both the early and late alpine grazing seasons, the herds were managed according to extensive farming methods, with fresh grass from pasture being the main feeding resource coupled with only limited concentrate supplementations. On the contrary, in autumn and winter the herds were mainly fed with conserved forages (hay) and remarkable higher amounts of concentrates if compared to those supplied in the summer months. Such differences in the feeding regimen applied at farm level in the periods of production under analysis explain the observed significant variations in the FA compositions between the two summer grazing seasons on one hand and the indoor months on the other hand. Fresh grass, particularly from alpine pastures, has been reported to consistently ameliorate the FA profile of milk and dairy products from ruminants, by lowering the levels of medium chain hypercholesterolemic saturated lauric (C12), myristic (C14), and palmitic (C16) acids, and by contemporarily raising the amounts of peculiarly beneficial FAs such as oleic, *trans*-vaccenic, rumenic, and α-linolenic acids (van Dorland et al. 2006). Fresh grass is an important source of lipids in the ruminants’ diet and, if compared to hay, has been reported to contain higher amounts of ALA, the latter usually representing more than 50% of total FAs (Clapham et al. 2005). Alpha-linolenic acid is extensively biohydrogenated within the rumen, leading to the formation of intermediate products (TVA and RA) characterized by

putative beneficial effects for human health (Bauman and Lock 2010). Similar results to those obtained in the current study have been previously obtained by other authors, who reported significant seasonal (summer *versus* winter) changes in the FA compositions of milk and dairy products from ruminants, mainly as a consequence of the nature and composition of the feedstuffs fed to the animals (among others, Revello Chion et al. 2010; Abilleira et al. 2009). Our results show that many detected fatty acids (including ALA, TVA, and RA) could be particularly useful for the discrimination of Asiago d'Allevo PDO cheeses produced during the grazing and indoor seasons. However, the same fatty acids do not prove useful for the discrimination of Asiago cheeses produced during the early and late summer grazing seasons.

The results obtained showed that terpenoids were very effective in differentiating Asiago PDO cheeses not only between the summer grazing and the indoor seasons but also between the early and late summer grazing periods. It is known that, as with green grass, hay prepared from natural grasslands can also provide terpenoids (Viallon et al. 1999). The differences in terpenoids observed between the summer and autumn/winter Asiago cheese samples could be consequently ascribed to a diverse terpenoidic composition of hay and grazed grass vegetation types. Moreover, it has also been reported that even though the haymaking process does not significantly affect the terpene profile of milk, the amount of terpenoids in milk can be strongly reduced (Fedele et al. 2007).

The observed increase of the content of some terpenoids during the grazing season (early *versus* late summer) could probably be related to the accumulation of plant

secondary metabolites. The sole presence of dihydrocarveol isomer 1 and unidentified sesquiterpene 1 in the late summer could be explained in the same way, as well as the evidence that a lot of monoterpenoids (α- and β-pinene, γ-terpinene, linalool, carvone, and myrtenol) did not show significantly different amounts between the early summer and the indoor seasons.

In order to trace Asiago d'Allevo PDO cheese according to its season of production, the most effective terpenoids were found to be camphene, δ-3-carene, and cedrane. In fact, they all significantly differed among the three production periods analysed, showing their maximum levels in cheeses produced during the late summer grazing season. Among other detected terpenoids, α-thujene and β-ionone were found to be discriminative of early summer cheeses. A thick group of compounds (α-pinene, β-pinene, myrtenol, dihydrocarveol isomer 1, and unidentified sesquiterpene 1) allowed a good discrimination of late summer cheeses. Finally, the two menthone isomers, terpinen-4-ol, and β-caryophyllene allowed a good discrimination of cheeses produced during the indoor season.

Sesquiterpenoids have been reported as being preferable to monoterpenoids for traceability purposes (Engel et al. 2007). If considering the main terpenoid groups, the results obtained in the current study seem to corroborate such a finding. In fact, even though the total monoterpenoids were by far more abundant in the Asiago cheese samples than the total sesquiterpenoids in all three periods in question, the total sesquiterpenoids were found to better discriminate the samples according to their season of production.

Some monoterpenoid compounds, such as β-myrcene, limonene, cineol, terpinolene, camphor, bornyl acetate, and α-terpineol, did not differ among the three periods of production. They were consequently ineffective in the attempt to trace the Asiago PDO cheese produced in different seasons.

The current study provided the first investigation into the possible role of a chemical class of volatiles, denominated "miscellaneous" category, as biochemical markers to be used for dairy products. These plant-derived compounds are C13-norisoprenoidic volatiles such as β-ionone and methyl dihydrojasmonate, or benzene derivatives such as methyl salicylate. The latter is biosynthesized in plants by carboxyl methyltransferase from salicylic acid, which in turn derives from phenylalanine (Effmert et al. 2005). Methyl dihydrojasmonate is responsible for flowery aroma and it has been reported as a linoleic acid derived molecule (Barreto et al. 2011), while β-ionone is a ubiquitous constituent of many vegetables and fruits (Winterhalter and Rouseff 2001). The results obtained in this study showed that, even though the total miscellaneous compounds did not vary significantly among the three periods of production, β-ionone was detected only in the Asiago cheese samples produced in the early summer grazing period. β-ionone has already been detected in dairy products, and it has recently been found useful in differentiating milk and cheeses produced under extensive (with pasture-fed dairy cows) or intensive (with cows fed total mixed rations) farming conditions (Belviso et al. *in press*). This norisoprenoidic volatile could be consequently considered a promising compound to be used for traceability purposes in the dairy sector.

Contrary to both fatty acid and terpenoidic compositions, the response obtained by the gas sensors equipment used in this study did not appear to be useful in classifying Asiago d'Allevo PDO cheeses according to their season of production. The same EN used in the present study was successfully applied in order to differentiate milk collected from dairy cows grazing on two different alpine vegetation types (Falchero et al. 2009). In strictly defined and controlled experimental conditions, these authors reported good discrimination ability of the model (LDA classification rate: >90%) highlighting actual applicability of this instrument to milk samples.

Concerning Asiago PDO cheese, Benedetti and Mannino (2007) first analyzed it with an EN. These authors submitted samples of "Pressato" (20 days of ripening) and "d'Allevo" (3-12 months of ripening) Asiago PDO cheeses to gas sensors measurements. Both fresh and ripened samples were produced in winter and summer months. The EN was able to identify some outliers (identically recognized as anomalous samples with sensory analysis by experts) probably due to unusual fermentations. However, the instrument detected similar headspace compositions for the ordinary samples and no discrimination according to the season of production (summer *versus* winter) was then possible, neither for the "Pressato" nor for the "d'Allevo" Asiago cheeses.

Although several studies demonstrated the ability of ENs to discriminate aged cheeses with different geographical origin at different maturation stages (Pillonel et al. 2003) and even at the same ripening age (Schaller et al. 1999), O'Riordan and Delahunty (2003) also suggested that the MOS sensors-based EN used in their

study gradually deteriorated in its accuracy in classifying Cheddar cheeses with the progression of cheese maturation. The results obtained in the present study support those reported by Benedetti and Mannino (2007) and O'Riordan and Delahunty (2003). Differences detected by EN analysis in flavor and volatile compounds of fresh cheeses due to animal diet and milk microflora could be progressively attenuated due to complex microbiological and enzymatic processes that occur during ripening.

CONCLUSIONS

The results obtained in the present *on-farm* research showed that both fatty acids and terpenoids can be considered suitable biomarkers to be used as tracers in discriminating Asiago d'Allevo PDO cheeses according to their season of production. Fatty acids and terpenoids, in fact, were found to be valuable as chemical fingerprint for the characterization of the dairy cows' feeding regimen. It is worth mentioning that information obtained from both fatty acids and terpenoidic data provided more comprehensive and precise information. Fatty acids data were in fact very useful in discriminating summer from autumn/winter cheeses, while terpenoids proved to be very useful also in discriminating cheeses produced during the early and late summer grazing seasons. On the contrary, the use of a MOS sensors-based electronic nose on Asiago d'Allevo cheese did not seem to provide helpful information, producing an unsatisfactory classification rate of the collected samples regarding their period of production.

The obtained results suggest the usefulness of fatty acids and terpenoids as biomarkers for the traceability of cheeses originating from PDO areas, due to the strong link existing between the animals' diet and the chemical composition of ruminant-derived dairy products.

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Table 2 Terpenoidic composition (normalized arbitrary area units × 10^4) of Asiago d'Allevo PDO cheese manufactured during the early and late summer alpine grazing seasons and during the autumn/winter indoor season (mean±SE)

Fig. 1 PCA score plot based on gas sensors responses of Asiago d'Allevo PDO cheese according to the season of production: early alpine grazing season (Jun-Jul) (■), late alpine grazing season (Aug-Sep) (○), and autumn/winter indoor season (Oct-Feb) (▲)

Table 1 Fatty acid composition (g·100g^{-1} FAMEs) of Asiago d'Allevo PDO cheese manufactured during the early and late summer alpine grazing seasons and during the autumn/winter indoor season.

	Early alpine grazing season (Jun-Jul)	Late alpine grazing season (Aug-Sep)	Indoor season (Oct-Feb)	SEM	*P*
C4	2.14	1.96	2.13		ns
C6	1.72^{b}	1.64^{b}	1.88^{a}		**
C8	1.13^{b}	1.06^{b}	1.28^{a}		***
C10	2.27^{b}	2.14^{b}	2.78^{a}		***
C10:1 *c*9	0.25^{b}	0.26^{b}	0.29^{a}		***
C12	2.49^{b}	2.43^{b}	3.12^{a}		***
C13	0.14^{b}	0.15^{b}	0.18^{a}		***
C14 *iso*	0.15	0.18	0.18		ns
C14	9.39^{b}	9.46^{b}	10.78^{a}		***
C15 *iso*	0.31^{b}	0.37^{a}	0.32^{ab}		*
C15 *aiso*	0.60	0.66	0.58		ns
C14:1 *c*9	0.75^{c}	0.83^{b}	0.92^{a}		***
C15	1.07	1.16	1.13		ns
C16 *aiso*	0.28	0.33	0.32		ns
C16	25.88^{b}	25.92^{b}	29.54^{a}		***
C17 *iso*	0.13	0.11	0.13		ns
C17 *aiso*	0.61^{a}	0.60^{a}	0.47^{b}		***
C16:1 *c*7	0.30	0.29	0.25		ns
C16:1 *c*9	1.78	1.70	1.84		ns
C17	0.76	0.74	0.70		ns
C17:1	0.27^{a}	0.24^{a}	0.18^{b}		***
C18	12.18	12.39	11.61		ns
C18:1 *t*11 (TVA)	4.46^{a}	4.52^{a}	2.38^{b}		***
C18:1 *t*9	0.55	0.47	0.68		ns
C18:1 *c*9	24.32^{a}	24.12^{a}	21.43^{b}		***
C18:1 *c*11	0.57^{a}	0.57^{a}	0.46^{b}		**
C18:1 *c*12	0.18	0.18	0.25		ns
C18:1 *c*13	0.44^{a}	0.45^{a}	0.35^{b}		***
C18:2 *c*9*c*12 (LA)	2.37	2.49	2.29		ns
C20	0.17^{b}	0.20^{a}	0.17^{b}		***
C20:1	0.92^{a}	0.94^{a}	0.68^{b}		***
C18:3 *c*9*c*12*c*15 (ALA)	0.02^{a}	0.02^{a}	0.01^{b}		**
C18:2 *c*9*t*11 (RA)	1.37^{a}	1.42^{a}	0.67^{b}		***
Σ SFA	61.44^{b}	61.52^{b}	67.34^{a}		***

Σ MUFA	34.80[a]	34.56[a]	29.70[b]	***
Σ PUFA	3.77[a]	3.92[a]	2.97[b]	***
HSFA[x]	37.77[b]	37.81[b]	43.44[a]	***

Abbreviations: FAME, fatty acid methyl ester; *c*, *cis*; *t*, *trans*; TVA, *trans*-vaccenic acid; LA, linoleic acid; ALA, α-linolenic acid; RA, rumenic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; HSFA, hypercholesterolemic saturated fatty acids.

Probability: * P≤0.05; ** P≤0.01; *** P≤0.001; ns, not significant (P>0.05). Different letters within rows indicate statistically significant differences between groups.

[x] Calculated as C12:0+C14:0+C16:0.

Table 2 Terpenoidic composition (normalized arbitrary area units) of Asiago d'Allevo PDO cheese manufactured during the early and late summer alpine grazing seasons and during the autumn/winter indoor season (mean±SE).

	Early alpine grazing season (Jun-Jul)	Late alpine grazing season (Aug-Sep)	Indoor season (Oct-Feb)	*P*
Monoterpenoids				
α-Pinene	0.282 ± 0.140 ^{b}	0.952 ± 0.372 ^{a}	0.090 ± 0.024 ^{b}	***
α-Thujene	0.070 ± 0.054	0.002 ± 0.002	nd	**
Camphene	0.161 ± 0.104 ^{b}	0.455 ± 0.213 ^{a}	0.011 ± 0.007 ^{c}	***
β-Pinene	0.589 ± 0.239 ^{b}	1.174 ± 0.426 ^{a}	0.108 ± 0.024 ^{b}	***
Sabinene	0.179 ± 0.103 ^{a}	0.096 ± 0.042 ^{ab}	0.008 ± 0.004 ^{b}	*
δ-3-Carene	0.015 ± 0.006	0.025 ± 0.017	nd	*
β-Myrcene	0.067 ± 0.039	0.038 ± 0.017	0.027 ± 0.014	ns
Limonene	1.299 ± 0.389	0.738 ± 0.119	0.509 ± 0.117	ns
Cineol	0.019 ± 0.005	0.138 ± 0.037	0.131 ± 0.049	0.08
γ-Terpinene	0.233 ± 0.141 ^{a}	0.023 ± 0.015 ^{b}	0.024 ± 0.010 ^{ab}	*
p-Cymene	1.087 ± 0.451 ^{a}	0.820 ± 0.179 ^{ab}	0.414 ± 0.123 ^{b}	*
Terpinolene	0.109 ± 0.075	0.003 ± 0.002	0.011 ± 0.005	ns
Menthone1	nd	nd	0.476 ± 0.190	-
Menthone2	nd	nd	0.093 ± 0.059	-
Camphor	0.757 ± 0.283	0.833 ± 0.205	0.361 ± 0.112	ns
Linalool	1.565 ± 0.439 ^{ab}	2.058 ± 0.547 ^{a}	0.749 ± 0.178 ^{b}	*
Bornyl acetate	0.053 ± 0.017	0.060 ± 0.021	0.190 ± 0.090	ns
α-Terpineol	2.396 ± 0.543	3.660 ± 0.918	2.030 ± 0.490	ns
Terpinen-4-ol	0.480 ± 0.184 ^{a}	0.457 ± 0.100 ^{a}	0.084 ± 0.027 ^{b}	***
Carvone	0.277 ± 0.130 ^{ab}	0.366 ± 0.117 ^{a}	0.029 ± 0.013 ^{b}	**
Myrtenol	0.316 ± 0.194 ^{b}	1.534 ± 0.423 ^{a}	0.041 ± 0.011 ^{b}	***
Geranyl acetone	5.135 ± 1.332 ^{a}	4.948 ± 1.007 ^{ab}	1.515 ± 0.216 ^{b}	*
Dihydrocarveol is.1	nd	0.120 ± 0.071	nd	-
Monoterpene ni1	0.007 ± 0.004	0.006 ± 0.004	nd	ns
Sesquiterpenoids				
β-Caryophyllene	0.122 ± 0.035 ^{a}	0.442 ± 0.182 ^{a}	0.036 ± 0.011 ^{b}	*
Cedrane	0.073 ± 0.030 ^{b}	0.584 ± 0.156 ^{a}	0.022 ± 0.014 ^{c}	***
Sesquiterpene ni1	nd	0.006 ± 0.004	nd	-
Sesquiterpene ni2	0.021 ± 0.010	0.095 ± 0.051	0.007 ± 0.005	ns
Miscellaneous				
Methyl salicylate	0.114 ± 0.064	0.135 ± 0.059	0.077 ± 0.036	ns
β-Ionone	0.015 ± 0.011	nd	nd	-

Methyl dihydrojasmonate	0.876 ± 0.234	0.831 ± 0.166	0.750 ± 0.180	ns
Σ Monoterpenoids	15.276 ± 3.508	18.507 ± 3.950	6.900 ± 0.984	0.10
Σ Sesquiterpenoids	0.216 ± 0.063 [b]	1.127 ± 0.337 [a]	0.065 ± 0.025 [c]	***
Σ Miscellaneous	1.005 ± 0.250	0.967 ± 0.185	0.826 ± 0.178	ns

Abbreviations: nd, not detected; is., isomer; ni, not identified.

Probability: * P≤0.05; ** P≤0.01; *** P≤0.001; the P-value is shown if, although being not significant, it shows a tendency (P≤0.10); ns, not significant (P>0.10). Different letters within rows indicate statistically significant differences between groups. The statistical analysis was not performed for menthone 1, menthone 2, dihydrocarveol is.1, sesquiterpene ni1, and β-ionone since these variables were not detected in the samples belonging to two of the three groups.

Fig. 1 PCA score plot based on gas sensors responses of Asiago d'Allevo PDO cheese according to the season of production: early alpine grazing season (Jun-Jul) (■), late alpine grazing season (Aug-Sep) (○), and autumn/winter indoor season (Oct-Feb) (▲)